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NEW METHODS

Title of Invention

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Applicant(s) for DO/US

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To the United States Designated Office (DO/US):

- I. Accompanying this transmittal letter are certain items which are required under 35 U.S.C. 371 in order that United States National processing of the above identified International application may commence:
- (X) at the expiration of the applicable time limit under PCT Articles 22 and 39(1) according to the provisions of 35 U.S.C. 371(b).
- () as soon as possible upon receipt of this express request under 35 U.S.C. 371(f).

1. The U.S. National fee [35 U.S.C. 371(c)(1)]

a. () was previously transmitted by applicant on (date)_____.

b. (X) is submitted herewith as follows:

FOR	NO. FILED	NO. EXTRA	SMALL ENTITY			OTHER THAN SMALL ENTITY	
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Basic Fee	(USPTO NOT ISA OR IPEA)		////	\$485	or	////	\$970
Total Claims	-20 =	--	x 9 =		or	x18 =	\$
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i. (X) A check in the amount of **\$1464** is enclosed.

ii. () Please charge the filing fee, multiple dependent claim fee (if applicable), excess independent claims fee (if applicable), and excess total claims fee (if applicable) to **Deposit Account No. 23-1703**.

iii. (X) The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to **Deposit Account No. 23-1703**. A duplicate copy of this sheet is enclosed.

(iv) () The filing fee is not enclosed.

2. A copy of the International application as filed [35 U.S.C. 371(c)(2)]:

a. (X) is transmitted herewith.

b. () is not required as the application was filed with the United States Receiving Office.

c. () has been transmitted

- i. () by the International Bureau. Date of mailing of the application (from form PCT/IB/308): _____ A copy of form PCT/IB/308 is enclosed.
- ii. () by applicant on (date) _____.
3. A translation of the International application into the English language [35 U.S.C. 371(c)(2)]:
- a. () is transmitted herewith.
- b. (X) is not required as the application was filed in English.
- c. () was previously transmitted by applicant on (date) _____.
4. Amendments to the claims of the International application under PCT Article 19 [35 U.S.C. 371(c)(3)]:
- a. () are transmitted herewith.
- b. () have been transmitted
- i. () by the International Bureau. Date of mailing of the amendments (from form PCT/IB/308): _____.
- ii. () by applicant on (date) _____.
- c. (X) have not been transmitted as
- i. () no notification has been received that the International Searching Authority has received the Search Copy.
- ii. () the Search Copy was received by the International Searching Authority but the Search Report has not yet issued. Date of receipt of Search Copy (from form PCT/ISA/202): _____.
- iii. () applicant chose not to make amendments under PCT Article 19. Date of mailing of Search Report (from form PCT/ISA/210): _____.

iv. (X) the time limit for the submission of amendments has not yet expired. The amendments or a statement that amendments have not been made will be transmitted before the expiration of the time limit under PCT Rule 46.1.

5. A Translation of the amendments to the claims under PCT Article 19 [35 U.S.C. 371(c)(3)]:

a. () is transmitted herewith.

b. () is not required as the amendments were made in the English language.

c. (X) has not been transmitted for reasons indicated at point I.4.b. or c. above.

6. An executed declaration for patent application of the inventor [35 U.S.C. 371(c)(4)] complying with 35 U.S.C. 115:

a. () was previously submitted by applicant on (date)

b. (X) is submitted herewith;
and such oath or declaration

i. (X) is attached to the application.

ii. (X) identifies the application and any amendments under PCT Article 19 which were transmitted as stated in points 1.2.b. or c. and 1.4. and states that they were reviewed by the inventor as required by 37 CFR 1.70.

c. () will be submitted subsequently.

II. Concerning other documents:

1. An International Search Report or Declaration under PCT Article 17(2)(a):

a. () has been transmitted by the International Bureau. Date of mailing (from form PCT/IB/308): _____. A copy of form PCT/IB/308 is enclosed

b. () is not required as the application was searched by the United States International Searching Authority.

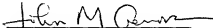
- c. ☐ A copy of the International Search Report is transmitted herewith.
- d. ☐ has been submitted by applicant on (date) _____.
2. A Statement of prior art under 37 CFR 1.97 and 1.98:
- a. ☐ is transmitted herewith including copies of the references cited on the attached form PTO-1449. Also enclosed is a copy of the International-Type Search Report (PCT/ISA/201/SE), issued in the Swedish priority application.
- b. ☐ will be transmitted within THREE MONTHS of the date of submission of requirements under 35 U.S.C. 371(c).
- c. ☐ was previously submitted by applicant on _____, in application serial no. _____.
3. (X) An Assignment is transmitted herewith for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
- a. ☐ Please charge the \$40.00 assignment recordation fee to Deposit Account No. 23-1703.
- b. (X) Enclosed is a check in the amount of \$40.
4. **Other document(s) or information included:**
- Copy of PCT/RO/101 - The PCT Request Form;
 - Six (6) sheets of drawings;
 - Four (4) pages of sequence listing and a diskette containing a computer readable form of the sequence listing; and
 - Return postcard.

5. (X) Please amend the specification by inserting the following sentence before the first line:

This application is the National Stage of International Application No. PCT/SE00/00878, filed May 4, 2000.

Respectfully submitted,

22 August 2000
DATE


John M. Genova
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enclosures

ASTRA

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Title: **NEW METHODS**

Reference: H 2174-1 WO

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G/PRTS

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NEW METHODS

FIELD OF THE INVENTION

5 The present invention relates to nucleic acid molecules constituting GABA_B receptor 1 promoters P1a and/or P1b, and to methods for screening for compounds which are modulators of GABA_B receptor 1 transcription, said methods comprising the use of nucleic acid molecules constituting GABA_B receptor P1a and/or P1b promoters.

10 BACKGROUND

GABA_B receptor 1

15 GABA (γ-aminobutyric acid) is an endogenous neurotransmitter in the central and peripheral nervous systems. Receptors for GABA have traditionally been divided into GABA_A and GABA_B receptor subtypes. GABA_B receptors (for a review see Kerr, D.I.B. and Ong, J. (1995) Pharmac. Ther. vol. 67, pp.187-246) belong to the superfamily of G-protein coupled receptors. GABA_B receptor agonists are described as being of use in the treatment of CNS disorders, such as muscle relaxation in spinal spasticity, cardiovascular disorders, asthma, gut motility disorders such as irritable bowel syndrome and as prokinetic and anti-tussive agents. GABA_B receptor agonists have also been disclosed as useful in the
20 treatment of emesis (WO 96/11680) and reflux disease (WO 98/11885).

The cloning of the cDNA encoding the rat GABA_B receptors splice isoforms GABA_BR1a and GABA_BR1b is disclosed by Kaupmann et al. (1997) Nature, vol. 386, 239-246. The mature rat GABA_BR1b differed from GABA_BR1a in that the N-terminal 147 residues were
25 replaced by 18 different residues. It was presumed that the rat GABA_BR1a and -b receptor variants are derived from the same gene by alternative splicing.

The cloning of the cDNA encoding the human GABA_B receptor GABA_BR1b is disclosed in WO 97/46675.

The cloning of the human GABA_B receptor 1 gene and the elucidation of the exon-intron organization is in part or fully disclosed in PCT/SE98/01947, in EMBL HS271M21 (GenBank AL031983), EMBL AJ010170 to AJ010191, in Peters, HC et al., Neurogenetics 2; 47-54 (1998) and in Goei, VL et al. Biological Psychiatry. 44; 659-66 (1998). The human GABA_B receptor 1 gene consists of 23 exons, spanning over a distance of 30 kb (Figure 1). The elucidation of the gene organization revealed that the human GABA_BR1a and GABA_BR1b are splice variants encoded by a single gene. The GABA_BR1a and GABA_BR1b isoforms are differentially expressed, at least in the rat (Kaupmann et al. (1997) Nature, vol. 386, 239-246). The physiological consequences of multiple GABA_B receptor 1 splice isoforms has not yet been determined, but their existence constitute an opportunity for the development of specific pharmaceutical agents.

GABA_B receptor 2

Based on its homology with the mammalian GABA_BR1 cDNA, a second member of the GABA_B receptor family was identified (Jones, KA et al., Nature 396; 674-679 (1998), White, JA et al., Nature 396; 679-682 (1998), Kaupmann, K et al., Nature 396; 683-687 (1998), WO 99/20751). The corresponding protein, GABA_BR2, forms heteromers with GABA_BR1a and R1b, resulting in cell surface expression of a functional GABA_B receptor (Kuner, R et al. Science 283, 74-77 (1999)). At least in recombinant expression systems, GABA_BR1 and R2 coexpression is necessary for the formation of a functional GABA_B receptor. Jones et al. (Nature 396; 674-679 (1998)) disclosed that a GABA_BR1: GABA_BR2 stoichiometry of 1:1 is an optimal ratio for successful cell surface expression of a ligand binding and functional GABA_B receptor. Thus, modulating GABA_BR1 expression could alter the stoichiometry between GABA_BR1 and other interacting proteins and be a means to regulate signaling through GABA_B receptors and thereby interfere with various physiological processes.

Transcriptional regulation

Gene regulation is mediated by specific DNA elements in the promoter that directs binding of transcription factors, which thereby mediate transcription of the gene. Eukaryotic transcription factors can be divided in two main groups *i*) basal transcription factors that

interact with promoter sequences proximal to the start of transcription, thereby initiating transcription upon recruitment of RNA polymerase II and *ii*) transcription factors that bind to specific distal promoter elements, thereby modulating the transcription upon contact with the basal transcription machinery. The DNA sequence that directs the start of transcription in most eukaryotic genes is the TATA-box, which is often located approximately 30 base pairs upstream from the start of transcription. However, the TATA-box is not a prerequisite for initiation of transcription as there are many promoters, including the GABA_B R1 promoters described in this study, that lack a TATA-box. A fundamental physiological process in the eukaryotic organism is that cells can communicate with their environment and respond to extracellular stimuli through signaling molecules, such as hormones and growth factors. The final event for such signaling is the binding of transcription factors to specific distal promoter elements leading to for example up-regulated or tissue specific gene expression. Because of their regulatory role, signaling molecules are putative targets for screening of therapeutic agents. The presence of two distinct and differentially regulated promoters within the human GABA_B receptor 1 gene, disclosed in this patent application, makes it possible to screen for therapeutic agents selectively regulating expression of GABA_B receptor 1a and 1b-type splice isoforms.

Indications

Compounds which are modulators of GABA_B receptor 1 transcription are potentially useful in the treatment of disorders which are related to neurally-controlled physiological responses regulated by GABA_B receptors, e.g. CNS disorders such as muscle relaxation in spinal spasticity, Alzheimer's disease and other dementias, psychiatric and neurological disorders such as depression, anxiety and epilepsy, cardiovascular disorders, asthma, gut motility disorders such as irritable bowel syndrome, emesis and reflux disease. In some humans, the lower esophageal sphincter (LES) is prone to relaxing more frequently than in other humans. As a consequence, fluid from the stomach can pass into the esophagus since the mechanical barrier is temporarily lost at such times, an event hereinafter referred to as "reflux".

Gastro-esophageal reflux disease (GERD) is the most prevalent upper gastrointestinal tract disease. Current therapy has aimed at reducing gastric acid secretion, or by reducing esophageal acid exposure by enhancing esophageal clearance, lower esophageal sphincter tone and gastric emptying. The major mechanism behind reflux has been considered to depend on a hypotonic lower esophageal sphincter. However, recent research (e.g. Holloway & Dent (1990) Gastroenterol. Clin. N. Amer. 19, 517-535) has shown that most reflux episodes occur during transient lower esophageal sphincter relaxations (TLESR), i.e. relaxations not triggered by swallows. It has also been shown that gastric acid secretion usually is normal in patients with GERD. Consequently, there is a need for compounds which reduce the incidence of TLESR and thereby prevent reflux.

DESCRIPTION OF THE INVENTION

This invention relates to nucleic acid molecules constituting GABA_B receptor 1 promoters and fragments of said promoters. By GABA_B receptor 1 promoters is understood the nucleic acids sequences upstream of the ATG translation initiation codon of GABA_B receptor 1a of the GABA_B receptor 1 gene, designated P1a, and the nucleic acids sequences upstream of the ATG translation initiation codon of GABA_B receptor 1b of the GABA_B receptor 1 gene, designated P1b, as illustrated in Figure 1.

In the present context the term "promoter" is meant to include core promoter sequences proximal to the start of transcription and upstream promoter elements which bind constitutively active transcription factors, as well as distal promoter elements which bind specific transcription factors.

Accordingly, the present invention provides a nucleic acid molecule constituting a human GABA_B receptor 1 promoter P1a, or a functionally equivalent modified form thereof, or active fragments thereof. The present invention also provides a nucleic acid molecule constituting a human GABA_B receptor 1 promoter P1b, or a functionally equivalent

modified form thereof, or active fragments thereof. By a functionally equivalent modified form is understood nucleic acids modified from the original sequence that can bind transcription factors. By active fragments of the promoters is understood nucleic acid fragments that can bind transcription factors.

In preferred forms of the invention the said nucleic acid molecule is selected from:

- (a) a nucleic acid molecule comprising a nucleotide sequence set forth as SEQ ID NO: 1;
- (b) a nucleic acid molecule comprising a nucleotide sequence capable of hybridizing, under stringent conditions, to a nucleotide sequence complementary to the DNA molecule as defined in (a).

In other preferred forms of the invention the said nucleic acid molecule is selected from:

- (a) a nucleic acid molecule comprising a nucleotide sequence set forth as SEQ ID NO: 2;
- (b) a nucleic acid molecule comprising a nucleotide sequence capable of hybridizing, under stringent conditions, to a nucleotide sequence complementary to the DNA molecule as defined in (a).

In another preferred form of the invention the said nucleic acid molecule may be a nucleic acid molecule constituting a human GABA_B receptor 1 promoter P1a, or a functionally equivalent modified form thereof, or active fragments thereof, in combination with a nucleic acid molecule constituting a human GABA_B receptor 1 promoter P1b, or a functionally equivalent modified form thereof, or active fragments thereof.

In another preferred form of the invention the said nucleic acid molecule may be a nucleic acid molecule selected from:

- (a) a nucleic acid molecule comprising a nucleotide sequence set forth as SEQ ID NO: 1;
- (b) a nucleic acid molecule comprising a nucleotide sequence capable of hybridizing, under stringent conditions, to a nucleotide sequence complementary to the DNA molecule as defined in (a);

in combination with a nucleic acid molecule is selected from:

- (a) a nucleic acid molecule comprising a nucleotide sequence set forth as SEQ ID NO: 2;
(b) a nucleic acid molecule comprising a nucleotide sequence capable of hybridizing, under stringent conditions, to a nucleotide sequence complementary to the DNA molecule as defined in (a).

It should thus be understood that the nucleic acid molecules according to the invention is not to be limited strictly to molecules comprising the sequences set forth as SEQ ID : 1 and 2. Rather the invention encompasses nucleic acid molecules carrying modifications like substitutions, small deletions, insertions or inversions, which nevertheless have substantially the biochemical activity of the GABA_B receptor promoters 1a and/or 1b according to the invention. Included in the invention are consequently nucleic acid molecules, the nucleotide sequence of which is at least 95% homologous, preferably at least 96%, 97%, 98% or 99% homologous, with the nucleotide sequence shown as SEQ ID NO: 1 or 2 in the Sequence Listing.

The term "stringent hybridization conditions" is known in the art from standard protocols (e.g. Current Protocols in Molecular Biology, editors F. Ausubel et al., John Wiley and Sons, Inc. 1994) and could be understood as as stringent or more stringent than those defined by e.g. hybridization to filter-bound DNA in 0.5 M NaHPO₄, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at +65°C, and washing in 0.1xSSC / 0.1% SDS at +68°C.

It will be appreciated that the nucleic acid sequences shown in the Sequence Listing is only an example within a large but definite group of nucleic acid sequences, which will have the GABA_B receptor promoter activity.

In yet another aspect, the invention provides a vector transformed with a nucleic acid molecule of the present invention. The said vector can e.g. be a replicable expression vector, which carries a nucleic acid molecule according to the invention. In the present context the term "replicable" means that the vector is able to replicate in a given type of host cell into which is has been introduced. Examples of vectors are viruses such as

bacteriophages, cosmids, plasmids and other recombination vectors. Nucleic acid molecules are inserted into vector genomes by methods well known in the art.

Another embodiment of the present invention is an expression system comprising nucleic acid molecules encoding GABA_B receptor promoters P1a and/or P1b, or functionally equivalent modified forms, or active fragments thereof.

In preferred forms of this embodiment of the invention the said nucleic acid molecule is selected from: (a) a nucleic acid molecule comprising a nucleotide sequence set forth as SEQ ID NO: 1 and/or SEQ ID NO: 2 ; (b) a nucleic acid molecule comprising a nucleotide sequence capable of hybridizing, under stringent conditions, to a nucleotide sequence complementary to the polypeptide coding region of a DNA molecule as defined in (a).

In another preferred form of this embodiment of the invention the said nucleic acid molecule may be a nucleic acid molecule constituting a human GABA_B receptor 1 promoter P1a, or a functionally equivalent modified form thereof, or active fragments thereof, in combination with a nucleic acid molecule constituting a human GABA_B receptor 1 promoter P1b, or a functionally equivalent modified form thereof, or active fragments thereof.

In another preferred form of this embodiment of the invention the said nucleic acid molecule may be a nucleic acid molecule selected from:

(a) a nucleic acid molecule comprising a nucleotide sequence set forth as SEQ ID NO: 1; (b) a nucleic acid molecule comprising a nucleotide sequence capable of hybridizing, under stringent conditions, to a nucleotide sequence complementary to the DNA molecule as defined in (a);

in combination with a nucleic acid molecule is selected from:

(a) a nucleic acid molecule comprising a nucleotide sequence set forth as SEQ ID NO: 2; (b) a nucleic acid molecule comprising a nucleotide sequence capable of hybridizing, under stringent conditions, to a nucleotide sequence complementary to the DNA molecule as defined in (a).

The expression system may, in addition, comprise a reporter gene, the promoter and the reporter gene being positioned so that the expression of the reporter gene is regulated by the GABA_B receptor 1 promoters P1a and/or P1b. Suitable expression systems according to the invention are e.g. bacterial or yeast plasmids, wide host range plasmids and vectors derived from combinations of plasmid and phage or virus DNA. Furthermore, an origin of replication and/or a dominant selection marker can be present in the vector according to the invention. Suitable reporter genes that can be used for the construction of expression systems according to the invention are e.g. the firefly luciferase gene, the bacterial chloramphenicol acetyl transferase (CAT) gene, the β -galactosidase (β -GAL) gene, and the green fluorescent protein (GFP).

A further aspect of this embodiment of the invention is a host cell transfected with an expression system comprising nucleic acid molecules constituting GABA_B receptor promoters P1a and/or P1b, or functionally equivalent modified forms thereof, or active fragments thereof.

Suitable host cells are cells known to express GABA_B receptors or cells known to express transcription factors, which can influence the transcription of GABA_B receptors. Host cells transfected with DNA encoding specific transcription factors can preferably be used to study the interaction with defined transcription factors and the GABA_B receptor promoter.

Another embodiment of the invention is a method for the assay of GABA_B receptor promoter activity said method comprising the use of a host cell transfected with an expression system comprising nucleic acid molecules constituting GABA_B receptor promoters P1a and/or P1b, or functionally equivalent modified forms thereof, or active fragments thereof.

A further embodiment of the present invention is a method for the screening of compounds which are modulators of GABA_B receptor 1 transcription, said method comprising the use of nucleic acid molecules constituting GABA_B receptor P1a and/or P1b promoters.

Accordingly, the present invention provides a method for screening compounds which are modulators of GABA_B receptor 1 transcription, comprising the steps of: (a) transfecting a cell host with a suitable expression system comprising a nucleic acid molecule constituting human GABA_B receptor 1 promoter P1A, and/or a human GABA_B receptor 1 promoter P1B or functionally equivalent modified forms, or active fragments thereof coupled to a reporter gene; (b) contacting a test compound with the cell; and (c) determining whether the test compound modulates the level of expression of the reporter gene.

In one aspect of this embodiment of the invention it is provided a method of screening of compounds which are modulators of GABA_B receptor 1 transcription, wherein the cell host endogenously expresses GABA_B receptor 1.

In another aspect of this embodiment of the invention it is provided a method of screening of compounds which are modulators of GABA_B receptor 1 transcription, wherein the said cell host is further transfected with a suitable expression system comprising a nucleic acid molecule encoding one or more specific transcription factors. Preferably, the said transcription factors are selected from the group: CREB-1, CREB-2, CREM-1, ATF-1, ATF-2, ATF-3, ATF-4, Sp1, Sp2, Sp3, Sp4, AP-1, and AP-2.

A further embodiment of the invention is a transgenic non-human animal whose genome comprises an expression system comprising nucleic acid molecules constituting GABA_B receptor promoters P1a and/or P1b, or functionally equivalent modified forms thereof, or active fragments thereof, coupled to a reporter gene.

Such transgenic non-human mammals can be generated by insertion of DNA comprising GABA_B receptor promoters by microinjection, retroviral infection or other means well known to those skilled in the art, into appropriate fertilized embryos to produce a

transgenic animal (Hogan B. et al Manipulating the Mouse Embryo, A Laboratory Manual, Cold Spring Harbor Laboratory (1986)).

Accordingly, the present invention provides a method for the screening of compounds which are modulators of GABA_B receptor 1 transcription, comprising the use of a transgenic non-human animal whose genome comprises an expression system comprising nucleic acid molecules constituting GABA_B receptor promoters P1a and/or P1b, or functionally equivalent modified forms thereof, or active fragments thereof, coupled to a reporter gene, or tissues or cells isolated from such transgenic animals.

EXAMPLES

The following examples are preferred non-limiting examples embodying preferred aspects of the invention.

Example 1. Isolation and identification of human GABA_B R1 promoters P1a and P1b

Genomic DNA containing the human GABA_B receptor gene was isolated from human genomic libraries and genomic DNA. Human genomic libraries were obtained from Clontech (Palo Alto, CA, USA). The libraries were constructed from female leukocyte DNA (catalogue # HL1111J), cloned into λEMBL-3 vector. The average size of inserts are 16 kb and the number of independent clones are 1.7×10^6 . Human genomic DNA was obtained from Clontech (catalogue # 6550-1). In order to isolate recombinant phases containing exon and intron sequences of the human GABA_B receptor gene, 48 individual bacterial plates with a diameter of 150 mm and approximately 4×10^4 individual plaques per plate, were screened. The methods and solutions used were as described in the Library Protocol Handbook: General Procedures for the Hybridization of Lambda Phage Libraries w/DNA Probes (Clontech) with some modifications as will be apparent from the following.

The experiment was carried out essentially as follows. The numbers are given per plate basis. A sample of the phage library diluted in 0.1 ml sterile lambda diluent was prepared in order to obtain an estimated titer of 40,000 pfu (plaque forming units). A 0.6 ml LB-medium culture of the *E. coli* host strain K802 (obtained from Clontech) was infected with 40000 pfu recombinant phages for 15 minutes at 37°C. The culture was then mixed with 7 ml top agarose (6.5 g of agarose added per liter LB) and poured onto LB plates. The plates were incubated at 37°C for approximately 7 hours. The plates were then chilled at +4°C.

Plaque hybridization experiments were as follows. Membrane filters, Colony/Plaque Screen (DuPont, Wilmington, DE, USA), were placed onto the top of the plates for 3 minutes. For denaturation of DNA the filters were removed and floated in 0.5 M NaOH on a plastic wrap for 2 minutes, with the plaque side up. This step was repeated once to ensure efficient denaturation. Following neutralization the membrane filters were placed in 1M Tris-HCl pH 7.5, two times 2 minutes and allowed to dry.

To obtain probes for DNA hybridization screening of the membrane filters, a GABA_B receptor cDNA clone was digested with SacII and a 479 bp fragment, separated by agarose electrophoresis, excised and transferred to a polypropylene microcentrifuge tube.

Additional probes were obtained by PCR amplification of various regions of the GABA_B receptor cDNA. The isolated cDNA fragment was ³²P-labeled using Megaprime DNA labeling system (Amersham Pharmacia Biotech, Uppsala, Sweden) by the following procedure. Water was added at a ratio of 3 ml per gram of gel, and placed in a boiling water bath for 7 minutes to melt the gel and denature the DNA. A volume of DNA/agarose solution containing 25 ng of DNA was added to the labeling reaction, according to the supplier's instructions. Labeled nucleotides were removed from DNA labeling reactions using MicroSpinTM G-50 Columns (Amersham Pharmacia Biotech, Uppsala, Sweden).

The DNA hybridization reaction was performed under stringent conditions according to the method described below. The filter membranes were prehybridized at 65°C for at least 1 hour in a solution composed of 1% SDS, 1M NaCl, and 10% dextran sulfate using a

hybridization oven (Hybaid Ltd, Ashford, UK). Following prehybridization a solution containing denatured herring sperm DNA of a final concentration of 100 µg/ml and the ³²P-labeled DNA probe at a concentration <10 ng/ml (for optimal signal to background ratio) was added to the prehybridization solution and the membrane filters were incubated at 65°C for 10-20 hours. Following the removal of the hybridization solution the membrane filters were first washed in a 2xSSC (0.3M NaCl, 0.03M Na-citrate), 1% SDS solution two times for 5 minutes at room temperature. In the next step, the membrane filters were incubated at 60°C two times for 30 minutes each in the same solution. In a third step, the filters were washed two times at room temperature in 0.1xSSC. Finally, the membrane filters were placed on a sheet of filter paper with the DNA face up, and allowed to dry. The dried membrane filters were then exposed to X-ray films and autoradiographed.

Of the approximately 2x10⁶ individual plaques analyzed, four hybridizing plaques were detected and isolated. These four isolates were designated #GR1, #GR12, #GR13 and #GR41, respectively. After several rescreening experiments, the recombinant phage DNA was purified using Qiagen Lambda Midi Kit (Qiagen GmbH, Germany). The purified DNA was digested with SalI and the fragments representing the inserts were isolated by agarose electrophoresis.

The 16kb insert of isolate #GR13 was cloned into SalI digested linearized pUC19, resulting in the plasmid pAM364. The insert was analyzed by PCR, restriction mapping and hybridization to ³²P-labeled DNA fragments representing various regions of the GABA_B receptor cDNA.

The cloned fragment in the plasmid pAM364 was characterized by restriction enzyme mapping, using EcoRI, HindIII, PstI, and BamHI. The approximate positions of the exons and the approximate size of the introns were analyzed and determined by PCR-based exon-exon linking and agarose gel electrophoresis.

In order to facilitate nucleotide sequence analysis, 7 restriction sub-fragments derived from pAM364 were isolated and cloned individually into pUC19. The following strategy was

employed; by combining PCR primers located within the pUC19 sequence either upstream or downstream of the cloning site, with a PCR primer with defined orientation and specific for the GABA_B receptor derived subcloned fragment allowed the sequence determination.

5 The inserts were subjected to nucleotide sequence analysis. The nucleotide sequences for all subclones were determined using a Thermo Sequenase dye terminator cycle sequencing pre-mix kit (Amersham Pharmacia Biotech, Uppsala, Sweden). As primers for sequencing reactions specific oligonucleotides complementary to pUC19 or primers complementary to the human GABA_B receptor cDNA were used.

10 The sequence of the human GABA_B receptor gene fragment cloned in the plasmid pAM364 has previously been revealed (see PCT/SE98/01947). This genomic fragment was shown to contain the complete exons 1-11 and the complete introns 1-10 of the human GABA_B receptor gene as well as > 3kb sequence upstream of exon 1. The elucidation of
15 the gene organization revealed that the human GABA_BR1a and GABA_BR1b are splice variants encoded by a single gene.

In order to localize the putative human GABA_B receptor promoter, we investigated the genomic sequence for the presence of consensus sequences of known regulatory promoter
20 elements. To our surprise, we found that promoter elements, clustered in two regions: one region upstream of exon 1, and the other region in intron 5, just upstream of exon 6. We concluded that the human GABA_B receptor may be regulated by two independent promoters, and not by one single promoter as expected. The first putative promoter, denoted P1a (SEQ ID NO:1) and described in detail below, may regulate transcription of
25 GABA_BR1a-type splice variants and the second putative promoter, denoted P1b (SEQ ID NO: 2) and described in detail below, may regulate transcription of GABA_BR1b-type splice variants.

As indicated in the schematic representation of P1a and P1b (Figure 2), both putative
30 promoters lack a TATA box. However P1b has an initiator (Inr) element in position 4375-4381 which is located 24-30 bp upstream of the position corresponding to the 5' end of the

longest known cDNA isolated with "rapid amplification of cDNA ends" (RACE) PCR amplification. The Inr element may therefore direct the start of transcription from P1b. P1a contains neither a TATA or an Inr element and the transcription from R1a may therefore initiate from different start sites which is often the case in promoters lacking TATA boxes or Inr elements. Both P1a and P1b contain multiple GC rich regions at pos. 3009-3016, 3037-3044 and 3116-3123 in R1a and pos. 4080-4087, 4196-4204, 4241-4249 and 4272-4279 in R1b, which are potential binding site for the SP1 family of transcription factors. SP1 binding sites are often found in TATA lacking promoters where they often substantially contribute to transcription. In addition, to the indicated GC sequences in Figure 2 there are also other GC motifs that may function as SP1 binding sites. P1a further contains an activator protein-1 (AP-1) site at position 1497-1503. AP-1 sites are recognized by AP-1 transcription factors which consists of homodimers of members of the Jun family or Fos/Jun heterodimeric complexes. AP-1 complexes also interact, by protein-protein interactions, with members of the steroid receptor family and are therefore also targets for steroid receptor signaling. In addition to the GC motifs P1b also contain an activator protein-2 (AP-2) site at position 3844-3851 and a cAMP responsive element (CRE) at position 4308-4315. Especially the finding of a consensus CRE (TGACGTCA) is interesting as this promoter element is found in many genes regulated by cAMP which are bound and regulated by members of the ATF/CREB gene family. This sequence may therefore be an important target for cAMP mediated signaling via G-protein coupled receptors, including GABA_B receptors.

We conclude that transcription of the human GABA_B receptor gene may be regulated by two putative promoters, P1a and P1b, that may independently regulate expression of human GABA_B receptor 1a and 1b splice isoforms, respectively.

Example 2. Determination of GABA_B R1 promoter P1a and P1b activity

To experimentally determine if P1a and P1b indeed act as promoters, we fused these regions to a cDNA encoding firefly luciferase to be used as a reporter of promoter activity in transfected cells.

Reporter constructs containing R1a and R1b promoter fragments were generated by PCR using plasmid pAM 364, containing genomic sequence covering the promoter regions, as template. PCR reaction was performed by standard procedure (Perkin Elmer). Briefly, an initial denaturation at 95°C for 4 min was followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, elongation at 72°C for 1 min and finally a 7 min elongation at 72°C. In the sequence for primers used to generate promoter fragments (for details see Table 1. below), a Nhe I and Hind III endonuclease restriction enzyme site was introduced, in 5' and 3' primers respectively, to enable sub-cloning into Nhe I/Hind III digested pGL3-Basic luciferase reporter vector (Promega). Hence, complete promoter-reporter constructs (pAM440, pAM438 and pAM436) contain R1a and R1b promoter fragments (indicated size see table) fused to the luciferase reporter gene. Plasmid DNA were purified using Qiagen tip-100 columns according to suppliers instruction. Correct fragment insertion was verified by DNA sequencing.

Table 1. Nucleotide sequence of primers used to generate promoter fragments

Primer No.	Restriction site	Promoter sequence	Position	Sequence 5'-3'
1582	HindIII (AAG CTT)	SEQ ID NO: 1	3440-3424	AAG CTT CTC GGC GCG CGG GCC CG
1583	NheI (GCT AGC)	SEQ ID NO: 1	2341-2362	GCT AGC CAA GAG CTT CTG GAG CCG
1584	NheI (GCT AGC)	SEQ ID NO: 1	720-741	GCT AGC TGT TAC ATG CAG AGC AAT C
1585	HindIII (AAG CTT)	SEQ ID NO: 2	4439-4421	AAG CTT CCT ACG GCC CCC GCG
1586	NheI (GCT AGC)	SEQ ID NO: 2	3321-3340	GCT AGC GCG CAC TGC AAT GCC CTC

To determine putative promoter activity, the reporter gene constructs were introduced into mammalian cells by transfection. In this study the cell line ND7/23 (ECACC Ref No: 92090903) was used. ND7/23 is a hybrid cell line originating from a mouse neuroblastoma (N18tg2) fused by PEG to a rat dorsal root ganglion neuron cell line. This cell line was chosen since it express functional GABA_B receptors as evidenced by radioligand binding studies. Cells were cultured in a 1:1 mixture of Dulbecco's modified medium (DMEM) and Ham's F12 medium supplemented with 10% (v/v) fetal bovine serum (FBS). Cells were grown at 37°C in an atmosphere of 5% CO₂. ND7/23 cells (4×10^5) were transfected using the DMRIE-C reagent according to manufacturer's protocol (Gibco). Briefly, cells were seeded in 6 well tissue culture plates (Nunc) the day before transfection. Next day, 2µg of promoter-reporter construct and 1µg of transfection control construct (pSV-β-Galactosidase, Promega) was mixed with 0.5 ml Optimem media (Gibco). DNA containing media was then mixed with an equal volume (0.5ml) of Optimem containing 4µl of DMRIE-C reagent and the combined mixture was then incubated for 45 min. After incubation, the transfection mixture was added to the cells, which were washed with Optimem media just prior to addition of transfection mixture. Following 5h incubation at 37°C, an equal volume of 1:1 mixture of Dulbecco's modified medium (DMEM) and Ham's F12 medium supplemented with 20% (v/v) fetal bovine serum (FBS) was added to the cells. Cells were incubated for 24h at 37°C with or without cAMP enhancing supplement as indicated in Figure 3. Before cell harvest, cells were washed in PBS (7.6 mM Na₂HPO₄/NaH₂PO₄ pH 7.4 and 120 mM NaCl), then cell extracts were prepared by addition of 250µl reporter lysis buffer (Promega) to cells, followed by transfer of cell suspension to 1.5 ml tubes. Cells were further lysed by one round of freeze-thawing and 15s of vortex. Cell debris were removed by 2 minutes of centrifugation at 12 000 g. Luciferase activity in cell extracts was measured in a Luciferase Assay System (Promega), where 40µl of cell extracts was added to a 96-well plate (Maxisorp, Nunc) and mixed with 50µl of luciferase substrate. Luciferase activity was then measured in a LUMIstar (BMG Lab technologies). As internal control for transfection efficiency, β-Galactosidase (β-Gal.) activity was measured in 96-well plate (Maxisorp, Nunc) using a β-Galactosidase Enzyme

Assay (Promega) according to suppliers protocol. As seen in Figure 3, transient transfection of ND7/23 cells shows that both P1a and P1b has promoter activity as pAM438, pAM436 and pAM440 (Figure 3) result in reporter expression, while the pAM442 (vector control) has very low activity. In addition, this experiment demonstrates that especially reporter expression originating from pAM440 (R1b) can be induced by the cAMP activating agent forskolin. Moreover, forskolin induced expression may also be further enhanced in the presence of the phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine (IBMX) although this experiment does not show a significant difference.

In conclusion, this experiment demonstrate that P1a and P1b both have promoter activity and that the degree of activity can be modulated using the cAMP activating agent forskoline.

Example 3. Screening for substances modulating P1a and P1b activity

Modulating GABA_BR1 expression in a controlled way is a means to regulate signaling through GABA_B receptors which could be of significant therapeutic value for a variety of conditions. Particularly, the ability to specifically regulate expression of either 1a- or 1b-type GABA_B receptor splice isoforms could be of medical value if these isoforms could be attributed to specific conditions.

The experiment presented in Example 2 demonstrates that the use of P1a and P1b promoter/reporter constructions can be used to monitor GABA_B R1 expression in screens for therapeutic agents that can modify the expression of GABA_B receptor 1 isoforms. A screen for P1a and P1b modulating substances could be performed in ND7/23 cells as described in Example 2. A screen for P1a and P1b modulating substances could in addition be performed in any cell type with endogenous expression of GABA_B receptor 1 and 2 isoforms, in cells expressing recombinant GABA_B receptor 1 and 2 isoforms and in intact cells and in extract or fractions of cells expressing endogenous and recombinant proteins

modulating GABA_B receptor function. Such screens could furthermore be done in tissues and in living organisms.

Example 4. Functional analysis of GABA_B-R1 promoter fragments and modified forms

In order to identify functionally active promoter fragments of P1a and P1b, a deletion analysis of DNA fragments containing promoter fragments can be performed in two steps.

It is anticipated that P1a and P1b comprise active fragments that can mediate increased expression by binding of transcription factors that are activators as well as active fragments which can mediate decreased expression by binding of transcription factors that are repressors.

In the first step, promoter fragments mediating expression of a reporter gene when used in reporter constructs can be stepwise deleted or truncated to identify important regions.

Briefly, truncated or deleted promoter fragments are created by PCR using specific primers and the already identified promoter sequences as template. Reporter constructs (as exemplified in this application) comprising the deleted or truncated promoter fragments are then created. These reporter constructs can then be used in transfection experiments, as described above, to identify important regions of the promoters manifested in altered expression from constructs lacking active fragments compared to none-deleted constructs.

In the second step, the exact location and sequence of transcription factor binding sites within active fragments can be determined by PCR technique using specific primers harbouring desired mutations. Such promoter fragments, with specifically mutated promoter regions, can be used in transfection experiments, similar to those described above, to determine the exact sequence of functionally important nuclear factor binding sites within active promoter fragments, manifested in altered expression from constructs with mutated DNA sequence compared to none-mutated control constructs with equal size.

The above mentioned strategy can also be used to identify the specific active promoter fragments which are important for the effect on promoter activity of active substances identified when screening for therapeutic agents regulating GABA_B-R1 expression.

5 Example 5. Activity of GABA_B R1 promoter P1a and P1b fragments.

Reporter constructs containing P1a and P1b promoter fragments were generated by PCR as described in Example 2 and fused to the firefly luciferase reporter gene. The generated constructs are visualised in Figure 4.

10 Putative transcription initiation sites were identified at position 3207 in SEQ ID NO:1 for P1a and at position 4405 in SEQ ID NO:2 for P1b. The positions in the promoter region shown in Figure 4 were calculated setting the transcription initiation sites as position +1.

15 The generated promoter reporter constructs (see Figure 4) were used to transfect ND7/23 cells. As shown in Figure 4, deletion of P1a promoter region in between position -2549 bp and -361 caused an increase in expression, indicating putative repressor regions between position -2549 and -361. Moreover, when 175 bp of the 5' untranslated region were removed (compare the third and fourth P1a constructs from the top) there was no detected
20 difference in promoter activity and constructs with the shorter P1a 5'-untranslated region were therefore used for mutational analysis throughout the rest of the study. Additional deletions of the P1a promoter from position -361 to -46 caused a stepwise decrease of the expression that seem to correlate with removal of promoter regions containing GC elements (I-III). This indicate that 361 bp of the P1a promoter can confer optimal
25 expression and that promoter elements essential for R1a-type expression, including GC elements I-III are located between position -361 and -46. Similar to the P1a promoter, deletion of P1b promoter region from position -3238 bp to -390 caused an increase in expression, indicating putative repressor regions between -1084 and -390. Also, additional deletions of the P1b promoter from position -390 to -88 caused a stepwise decrease of the
30 expression that correlate with removal of promoter region GC elements (IV-VII) as well as the consensus CRE. Although there was still some promoter activity left when the P1b

promoter region was deleted down to position -88, the major promoter elements essential for R1b-type expression seem to be located between position -390 and -88. Comparison of P1a and P1b expression also indicates that, P1b mediated expression was higher (approximately 3-4 fold) than for P1a.

Example 6. Mutational analysis of P1a and P1b promoter element function

As shown in Figure. 4, reporter constructs -361 in P1a (short 5'UTR) and -390 in P1b conferred optimal expression of the respective promoters. In order to determine the importance of the promoter elements that was found within these regions, P1a (-361) and P1b (-390) constructs were used as templates to obtain reporter constructs with promoter regions mutated at specific sites. For the mutated promoter constructs, a 3 bp substitution was introduced by "quick change" site directed mutagenesis.

Site-directed mutagenesis: Mutant constructs were prepared with a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The mutagenic oligonucleotide primers are listed in Table 2. All constructs were verified by DNA sequencing.

Table 2. Oligonucleotide primers used in the mutagenesis experiments

Primer	Primer Sequences
1949 P R1b Cre Fwd	CGCCGCCCGTT TT GGTCAGAGCCCCCT
1950 P R1b Cre Rev	AGGGGGCTCTGAC CAA ACGGGCGGCG
1951 P R1a GCI Fwd	CTCTCTTCCCCC TAA CTGCCTTCCC
1952 P R1a GCI Rev	GGGAAGGCAG TTA AGGGGGGAAGAGAG

1953	P R1a GCII Fwd	GGCGGTCCAG TTAG GGGCTGGGATCC
1954	P R1a GCII Rev	GGATCCCAGCCCC TAA CTGGACCGCC
2051	P R1a GCIII Fwd	CCTCTCCACCGCCCC TAA CCACCGCGCTGTG
2052	P R1a GCIII Rev	CACAGCGCGGTGG TTA GGGCGGTGGAGAGG
2053	P R1b GCIVs Fwd	CCCCAGCTCCCGCCCC TAA CCCCACCCC
2054	P R1b GCIVs Rev	GGGGTGGGGG TTAG GGCGGGAGCTGGGG
2055	P R1b GCV Fwd	CGCTTCCCTCCCC TAA CCCTTCCTGCC
2056	P R1b GCV Rev	GGCAGGAAGGG TTAG GGGAGGGAAGCG
2057	P R1b GCVI Fwd	CCCTCCCCTCCCC TAA CTCCGACTGT
2058	P R1b GCVI Rev	ACAGTCGGAGG TTAG GGGAGGGGAGGG
2059	P R1b GCVII Fwd	CTCCGCCACCCC TAA CTCCTGGCAC
2060	P R1b GCVII Rev	GTGCCAGGAG TTAG GGGTGGGCGGAG
2146	P R1b GCIVd Fwd	CCCCAGCTCCC TAACTAA CCCCACCCC
2147	P R1b GCIVd Rev	GGGGTGGGGG TTAGTTAG GGAGCTGGGG

Bases that are labelled bold correspond to designed mutations

- 5 Obtained promoter constructs were then used to transfect ND7/23 cells. As shown in Figure 5, single point mutations of GC elements I, II and III reduced P1a expression to approximately 65-75% compared to wild-type, while double-mutations (I/II, I/III and II/III, respectively) resulted in a further decrease to approximately 55-60% of wild-type expression levels. When all three GC elements (I-III) were mutated, the reporter expression
- 10 was reduced to approximately one third (33%) of wild-type expression. Together, these results suggest that the three GC-elements found in P1a, all contribute to P1a mediated expression in a substantial and additive manner. However, the fact that one third of the P1a promoter activity still remains suggests that there are additional promoter elements within P1a contributing to P1a mediated expression.

In contrast to P1a, single mutations of the four P1b GC elements (IV, V, VI and VII) only caused small reductions of expression to approximately 75-90% compared to wild-type. The relatively modest contribution by the four P1b GC elements was also demonstrated by a promoter construct where all four GC elements (IV-VII) had been mutated. This construct retained 63% of P1b expression compared to the wild-type construct. However, when the P1b CRE consensus site was mutated (CRE), a dramatic reduction of P1b mediated expression (approximately 50%) was obtained. Moreover, when the CRE mutation was combined with mutations of each GC element respectively, a further reduction was observed. Most notably, the promoter construct containing a double mutation of the CRE and GC V (CRE/V) promoter elements resulted in a substantial reduction of P1b expression to approximately 26%, similar to the construct where all five P1b promoter elements were mutated (CRE/IV-VII) where 24% of the expression still remained compared to wild-type. Together these data demonstrates the absolute importance of the consensus CRE site for P1b expression, alone or in combination with the GC elements (IV, V, VI and VII), of which GC element V seems to contribute most. The fact that one fourth of P1b promoter activity still remains suggests that, as for P1a, there are additional promoter elements within P1b that may contribute to P1b mediated expression.

Example 7. Identification of factors interacting with the P1b CRE site

Electrophoretic mobility shift assays (EMSA)

Electrophoretic mobility shift assays (EMSA) can be used to identify nuclear factors that interact with P1a and P1b promoter elements. In an attempt to identify factors interacting with the P1b CRE site, we performed gelshift analysis with super-shift antibodies that recognise members of the CREB/ATF family of transcription factors. The DNA-binding reactions (12 μ l) were done as follows; 2-3 fmol 32 P-labeled double-stranded oligonucleotide corresponding to the P1b CRE site was mixed with 5 μ g crude nuclear extracts from ND7/23 cells and 1 μ g poly (dI-dC), 25mM Hepes (pH 7,9), 150 mM KCl, 5 mM dithiothreitol and 10% glycerol (Schneider et al 1986, Nucleic Acids Research. 14:1303-17). In the supershift lane (Figure 6) the ATF-1 p35/CREB-1 p43/CREM-1

reactive antibody (sc-270 from Santa Cruz Biotechnology, Santa Cruz, CA), was pre-incubated at room temperature for 20 min before addition of ^{32}P -labeled probe. After incubation at room temperature for 15 min, protein-DNA complexes were resolved on precasted DNA-retard gels (NovexTM) containing 6% polyacrylamide prepared with 0,5x TBE as gel buffer. Following electrophoresis, gels were dried and visualised by autoradiography.

As shown in Figure 6, addition of a monoclonal CREB/ATF supershift antibody (reactive with members of the ATF/CREB family such as ATF-1 p35, CREB-1 p43 and CREM-1 of mouse, rat and human origin) into the EMSA reaction mixture results in a distinct shift of the nuclear factor(s) that interacts with the P1b CRE site, while no super-shift was observed when the same antibody was added to other EMSA reactions containing other promoter elements (data not shown). This data suggests that the complex formed with the P1b CRE site contains a member of the CREB/ATF family.

Similar further studies can be performed in order to determine which factor(s) that interact with the various promoter elements in P1a and P1b.

Example 8. Use of recombinant transcription factors in the study of P1a and P1b promoter activities.

Cells with stably integrated or with transiently transfected P1a/P1b reporter constructs can be transfected with cDNA encoding specific transcription factors to make reporter cells suitable for investigations of P1a and P1b activities mediated by said transcription factors. Reporter cells can alternatively be generated by delivery of transcription factors into similar cells by various means such as e.g. microinjection and lipofection. Reporter cells can be utilised for screening of compounds, which are modulators of GABA_B receptor 1 transcription.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1. The human GABA_B receptor gene

The figure shows the organization of the human GABA_B receptor gene. Exons, represented by vertical squares/bars, are numbered (1-23). Translational start and stop sites are indicated by arrows. Location of human GABA_B receptor 1 promoters P1a (SEQ ID NO: 1) and P1b (SEQ ID NO: 2) are indicated. The extent of human GABA_B receptor genomic sequence cloned in plasmid pAM 364 is indicated by a horizontal bar.

Figure 2. Schematic representation of the P1a and P1b promoters

DNA fragments used to generate reporter constructs, corresponding to the positions in the P1a and P1b promoter sequence, are shown below each promoter. Putative promoter elements in each promoter are indicated. Arrows indicate putative positions for start of transcription.

Figure 3. Determination of GABA_B receptor 1 promoter P1a and P1b activity

ND7/23 cells (4×10^5) were transfected with promoter-luciferase constructs as described above. After transfection, cells were cultured in media without supplement (basal) or in the presence of Forskolin (10 μ M) or Forskolin (10 μ M) + 1-methyl-3-isobutylxanthine (0.125mM) for 24h. After incubation, cells were harvested and luciferase activity measured. Luciferase activity, minus background, is shown as arbitrary light units measured in 40 μ l of cell extracts. Relative values represent the mean \pm SEM of two individual experiments.

Figure 4: Deletion analysis of the GBR1 promoters in ND7/23 cells

ND7/23 cells (4×10^5) were transfected with P1a and P1b promoter-luciferase constructs as outlined above. After transfection, cells were cultured for 24h, harvested and luciferase activity was measured. Luciferase activity, minus background, is shown as arbitrary light units measured in 10 μ l of cell extracts. Relative values represent the mean \pm SEM of duplicate samples from at least three individual experiments.

Figure 5. Effect of P1a and P1b promoter element mutations on promoter activities.

ND7/23 cells (4×10^5) were transfected with wild-type and mutated P1a and P1b promoter-luciferase constructs as described above. The point mutations in the promoter constructs are outlined to the left. After transfection, the cells were cultured for 24h. After incubation, cells were harvested and luciferase activity measured. Luciferase activity, minus background, is shown as arbitrary light units measured in 10 μ l of cell extracts. Relative values represent the mean \pm SEM of duplicate samples from at least three individual experiments. Relative expression level of mutated constructs are indicated to the left with the P1a and P1b "wild-type" expression level set as 100%.

Figure 6. Identification of nuclear factors binding to the P1b consensus CRE site using CREB/ATF super-shift antibodies.

Nuclear extracts (5 μ g) from ND7/23 cells were incubated with double-stranded 32 P-labeled oligonucleotides containing the P1b consensus CRE site (sense: 5'-CGCCGCCCGTGACGTGACAGCCCCCT-3'). In lane 1, no antibody was added. In lane 2, a mouse monoclonal antibody (sc-270 Santa Cruz Biotechnology, Santa Cruz, CA) reactive with members of the ATF/CREB family such as ATF-1 p35, CREB-1 p43 and

CREM-1 was pre-incubated at room temperature for 20 min before addition of ^{32}P -labeled probe. The specific complex between nuclear factors and the CRE is indicated by a star and the super-shifted complex is indicated by two stars.

CLAIMS

1. A nucleic acid molecule constituting a human GABA_B receptor 1 promoter P1a, or a functionally equivalent modified form thereof, or an active fragment thereof.
2. A nucleic acid molecule according to claim 1 selected from:
 - (a) a nucleic acid molecule comprising a nucleotide sequence set forth as SEQ ID NO: 1;
 - (b) a nucleic acid molecule comprising a nucleotide sequence capable of hybridizing, under stringent conditions, to a nucleotide sequence complementary to the polypeptide coding region of a DNA molecule as defined in (a).
3. A nucleic acid molecule constituting a human GABA_B receptor 1 promoter P1b, or a functionally equivalent modified form thereof, or an active fragment thereof.
4. A nucleic acid molecule according to claim 1 selected from:
 - (a) a nucleic acid molecule comprising a nucleotide sequence set forth as SEQ ID NO: 2;
 - (b) a nucleic acid molecule comprising a nucleotide sequence capable of hybridizing, under stringent conditions, to a nucleotide sequence complementary to the polypeptide coding region of a DNA molecule as defined in (a).
5. A nucleic acid molecule comprising a nucleic acid molecule according to claim 1, in combination with a nucleic acid molecule according to claim 3.
6. A nucleic acid molecule comprising a nucleic acid molecule according to claim 2, in combination with a nucleic acid molecule according to claim 4.
7. A vector transformed with a nucleic acid molecule according to any one of claims 1 to 6.

8. A cultured cell host harboring a vector according to claim 7.
9. An expression system comprising a nucleic acid molecule constituting a human GABA_B receptor 1 promoter P1a, or a functionally equivalent modified form thereof, or an active fragment thereof.
10. An expression system according to claim 9, comprising a nucleic acid molecule selected from:
- (a) a nucleic acid molecule comprising a nucleotide sequence set forth as SEQ ID NO: 1;
- (b) a nucleic acid molecule comprising a nucleotide sequence capable of hybridizing, under stringent conditions, to a nucleotide sequence complementary to the polypeptide coding region of a DNA molecule as defined in (a).
11. An expression system comprising a nucleic acid molecule constituting a human GABA_B receptor 1 promoter P1b, or a functionally equivalent modified form thereof, or an active fragment thereof.
12. An expression system according to claim 11, comprising a nucleic acid molecule selected from:
- (a) a nucleic acid molecule comprising a nucleotide sequence set forth as SEQ ID NO: 2;
- (b) a nucleic acid molecule comprising a nucleotide sequence capable of hybridizing, under stringent conditions, to a nucleotide sequence complementary to the polypeptide coding region of a DNA molecule as defined in (a).
13. An expression system comprising a nucleic acid molecule according to claim 1, in combination with a nucleic acid molecule according to claim 3.
14. An expression system comprising a nucleic acid molecule according to claim 2, in combination with a nucleic acid molecule according to claim 4.

15. An expression system according to any one of claims 9 to 14, which, in addition, comprises a reporter gene.
- 5 16. An expression system according to claim 15, wherein the reporter gene is selected from:
- (a) the firefly luciferase gene,
 - (b) the bacterial amphenicol acetyl transferase (CAT) gene,
 - (c) the β -galactosidase (β -GAL) gene, and
 - 10 (d) the green fluorescent (GFP) gene.
17. An expression system according to claim 15 or claim 16, wherein the promoter and the reporter gene are positioned so that the expression system of the reporter gene is regulated by the GABA_B receptor 1 promoter.
- 15 18. An expression system according to any one of claims 9 to 17, wherein the said nucleic acid molecule is transformed in a vector.
19. An expression system according to claim 18, wherein said vector comprises an origin of replication and/or a dominant selection marker.
- 20 20. A host cell transfected with an expression system according to any one of claims 9 to 19.
- 25 21. A method of assay for GABA_B receptor promoter activity, comprising the use of a host cell according to claim 20.
22. A method for screening compounds which are modulators of GABA_B receptor 1 transcription, comprising the steps of:
- 30 (a) transfecting a cell host with a suitable expression system comprising a nucleic acid molecule constituting a human GABA_B receptor 1 promoter P1a, and/or a

human GABA_B receptor 1 promoter P1b, or functionally equivalent modified forms, or active fragments thereof coupled to a reporter gene;

(b) contacting a test compound with the cell; and

(c) determining whether the test compound modulates the level of expression of the reporter gene.

23. A method according to claim 22, wherein the said expression system is an expression system according to any one of claims 9 to 19.

24. A method according to claim 22, wherein the said the reporter gene is selected from:

(a) the firefly luciferase gene,

(b) the bacterial amphenicol acetyl transferase (CAT) gene,

(c) the β -galactosidase (β -GAL) gene, and

(d) the green fluorescent (GFP) gene.

25. The method according to claim 22, wherein the host cell endogenously expresses GABA_B receptor 1.

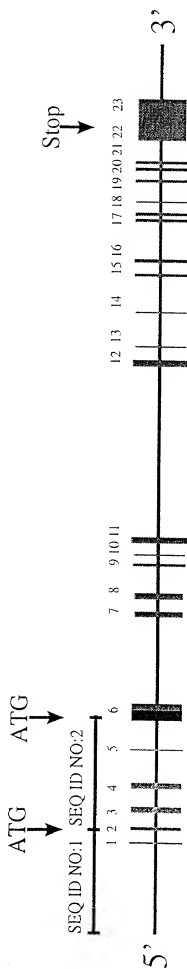
26. The method according to claim 22, wherein the host cell is further transfected with a suitable expression system comprising a nucleic acid molecule encoding one or more specific transcription factors.

27. The method according to claim 26, where the transcription factor is selected from the group: CREB-1, CREB-2, CREM-1, ATF-1, ATF-2, ATF-3, ATF-4, Sp1, Sp2, Sp3, Sp4, AP-1, and AP-2.

28. A transgenic non-human animal whose genome comprises an expression system comprising nucleic acid molecules constituting GABA_B receptor promoters P1a and/or P1b, or functionally equivalent modified forms thereof, or active fragments thereof, coupled to a reporter gene.

29. A transgenic non-human animal whose genome comprises an expression system according to any one of claims 9 to 19.
30. A method for the screening of compounds which are modulators of GABA_B receptor I transcription, comprising the use of a transgenic non-human animal according to claim 28 or claim 29.
31. A method for the screening of compounds which are modulators of GABA_B receptor I transcription, comprising the use of tissues or cells isolated from a transgenic non-human animal according to claim 28 or claim 29.

Fig. 1



pAM 364

F79.2

P1a (SEQ ID NO: 1)

? ?
↑ ↑



pAM438 (pos. 720) ————— (pos. 3440)

pAM436 (pos. 2341) ————— (pos. 3440)

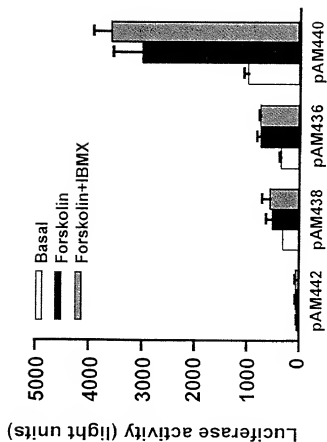
P1b (SEQ ID NO: 2)

? ?



pAM440 (pos. 3321) ————— (pos. 4439)

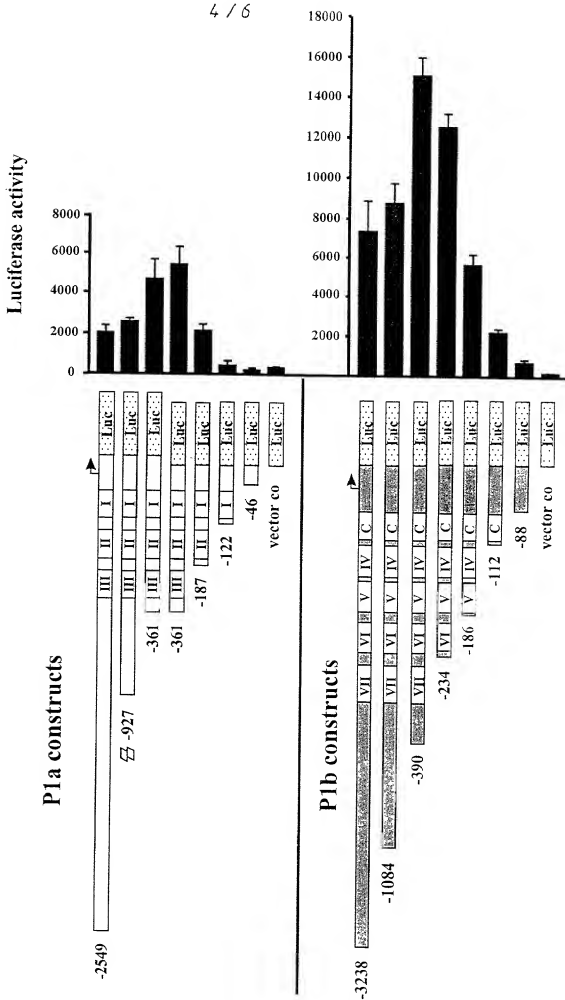
Fig. 3



Promoter Constructs

H-9-4

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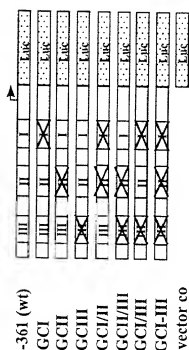


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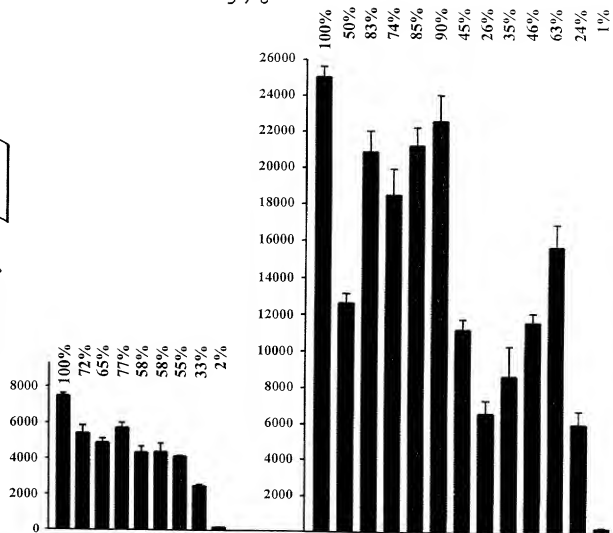
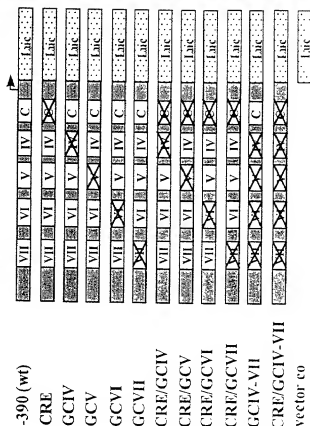
Fig. 5

Luciferase activity

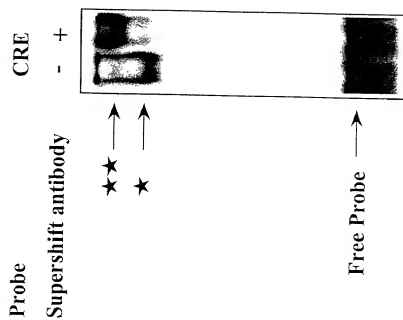
P1a constructs



P1b constructs



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Fig. 6

DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled NEW METHODS

the specification of which is attached hereto unless the following box is checked:

☒ was filed on 4 May 2000 as United States Application Number or PCT International Application Number SE00/00878 and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Priority Not Claimed

9901659-4
(Number)

Sweden
(Country)

6 May 1999
(Day/Month/Year Filed)



I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

(Application Number)

(Filing Date)

(Application Number)

(Filing Date)

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

(Application Number)

(Filing Date)

(Status -- patented, pending, abandoned)

(Application Number)

(Filing Date)

(Status -- patented, pending, abandoned)

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Dimitrios Drivas, Reg. No. 32,218; Cecilia O'Brien Lofters, Reg. No. 33,434; Warren S. Heit, Reg. No. 36,828; David Bender, Reg. No. 35,445; John M. Genova, Reg. No. 32,224; Richard J. Sterner, Reg. No. 35,372; Hans-Peter G. Hoffmann, Reg. No. 37,352; Leslie Morioka, Reg. No. 40,304; John Scheibeler, Reg. No. 35,346; Thelma A. Chen Cleland, Reg. No. 40,948; Jean E. Shimotake, Reg. No. 36,273; Jeff Oelke, Reg. No. 37,409; Chase Romick, Reg. No. 45,051; and Louis S. Silvestri, Reg. No. 45,108 of the firm of WHITE & CASE LLP, with offices at 1155 Avenue of the Americas, New York, New York 10036,

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believe to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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(given name, family name)

Anders Edlund

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Anders Edlund

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
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(given name, family name)

Jonas Ekstrand

Second inventor's signature



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Residence Address

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Citizenship

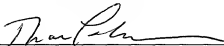
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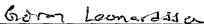
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